

## Successful Antiparasitic Treatment for Cysticercosis Is Associated with a Fast and Marked Reduction of Circulating Antigen Levels in a Naturally Infected Pig Model

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**Abstract.** *Taenia solium* cysticercosis is a common parasitic infection of humans and pigs. We evaluated the posttreatment evolution of circulating parasite-specific antigen titers in 693 consecutive blood samples from 50 naturally infected cysticercotic pigs, which received different regimes of antiparasitic drugs ( $N = 39$ , 7 groups), prednisone ( $N = 5$ ), or controls ( $N = 6$ ). Samples were collected from baseline to week 10 after treatment, when pigs were euthanized and carefully dissected at necropsy. Antigen levels decreased proportionally to the efficacy of treatment and correlated with the remaining viable cysts at necropsy (Pearson's  $p = 0.67$ ,  $P = 0.000$ ). A decrease of 5 times in antigen levels (logarithmic scale) compared with baseline was found in 20/26 pigs free of cysts at necropsy, compared with 1/24 of those who had persisting viable cysts (odds ratio [OR] = 76.7, 95% confidence interval [CI] = 8.1–3308.6,  $P < 0.001$ ). Antigen monitoring reflects the course of infection in the pig. If a similar correlation exists in infected humans, this assay may provide a minimally invasive and easy monitoring assay to assess disease evolution and efficacy of antiparasitic treatment in human neurocysticercosis.

### INTRODUCTION

Neurocysticercosis (NCC) is the infection of the central nervous system by the larval form of *Taenia solium*. NCC is the most important cause of late onset epilepsy worldwide and a growing problem in developed countries because of immigration from endemic zones.<sup>1,2</sup> Humans get infected by accidental ingestion of *T. solium* eggs excreted with the feces of human tapeworm carriers. The oncospheres are liberated from the eggs, cross the intestinal wall, and are carried by the circulatory system to almost any organ or tissue, where they develop as the larval stage or cysticercus.

Diagnosis is based principally on neuroimaging (magnetic resonance imaging and computed tomography) and available serological tests, mainly the immunoelectrotransfer blot (EITB), which detects specific antibodies.<sup>3–5</sup> Despite high sensitivity and specificity, the performance of antibody detection assays is suboptimal. In patients with single lesions or with calcified lesions only, the sensitivity decreases<sup>6</sup> or some individuals have specific antibodies in the absence of established infection (exposure only or aborted infections). Also, antibodies persist long after parasite clearance.<sup>7–10</sup> These drawbacks leave physicians dependent on imaging examinations, which are expensive and poorly available in endemic villages. In pigs, antibody detecting tests have similar limitations than in humans. They cannot distinguish between patent infection and exposure, although strong reactions may be associated with established infection and higher parasitic burden.<sup>11–13</sup> Detection of specific circulating antigens can reflect presence and burden of patent infection.<sup>14–17</sup> Cysticercosis antigen detection assays were reported as early as of 1989, using a monoclonal antibody-based enzyme-linked

immunosorbent assay (ELISA) directed to a secretory-excretory antigen from the related cestode *Taenia saginata*.<sup>18,19</sup> Brandt and others developed in 1992 an immunoglobulin M (IgM) monoclonal antibody-based assay,<sup>20</sup> later improved by using IgG monoclonal antibodies and pretreatment of the sera by trichloroacetic acid.<sup>21–23</sup> Antigen-ELISA has been widely used to detect cysticercosis in humans as well as in pigs.<sup>14,15,24–27</sup>

Using serial posttreatment serum circulating antigen levels in pigs, we assessed whether the changes in circulating antigen levels correlate with the response to antiparasitic treatment, and whether antigen levels at the time of necropsy correlate with the final infection burden.

### MATERIALS AND METHODS

**Study design and animals.** This study was conducted using serum samples of 50 pigs obtained from an original study cohort of 54 naturally *T. solium*-infected pigs.<sup>28</sup> These animals were bought from villages in the highly endemic Peruvian highlands and transported to veterinary facilities in Lima. The study was performed at the San Marcos School of Veterinary Medicine in Lima, Peru. Ethical approval was granted by the Animal Ethics Committee of the School of Veterinary Medicine, Universidad Nacional Mayor de San Marcos, Lima, Peru.

Pig infection was initially assessed using tongue examination (palpation of lingual viable nodules, implying the presence of active cysticercosis),<sup>29</sup> and confirmed by serology (serum EITB). Pigs were randomly assigned to nine different groups and each group was treated with an individual scheme, including albendazole (ABZ) (Zentel®; GlaxoSmithKline, Lima, Peru), praziquantel (PZQ) (Praziquantel; IDA foundation, Mumbai, India), three schemes of combined ABZ plus PZQ, oxfendazole (OFZ) (Synanthic®; American Home Products Corporation, Madison, WI), nitazoxanide (NTZ) (Colufase®; Roemmers, Buenos Aires, Argentina),

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prednisone (Nisona<sup>®</sup>; LUSA, Lima, Peru), and an untreated control group. All antiparasitic drugs were administered by oral route, initiating the same day, according the following schemes<sup>28</sup>: group 1 (AbPzPd)—ABZ 15 mg/kg/day in two doses (8 AM and 8 PM) for 7 days, three doses of PZQ 25 mg/kg each 2 hours starting at 8 AM, and prednisone at 0.5 mg/kg/day in single doses for 5 days; group 2 (½AbPzPd)—ABZ 7.5 mg/kg/day in two doses for 7 days, three doses of PZQ 25 mg/kg each 2 hours starting at 8 AM, and prednisone at 0.5 mg/kg/day in single doses for 5 days; group 3 (AbPz2Pd)—ABZ 15 mg/kg/day in two doses for 7 days, three doses of PZQ 25 mg/kg each 2 hours starting at 8 AM, and prednisone at 1 mg/kg/day in single doses for 5 days; group 4 (AbPd)—ABZ 15 mg/kg/day in two doses for 7 days plus prednisone at 0.5 mg/kg/day in single doses for 5 days; group 5 (OFZ)—OFZ 30 mg/kg/day single doses; group 6 (PZQ)—three doses of PZQ 25 mg/kg each 2 hours starting at 8 AM; group 7 (PDN)—prednisone at 0.5 mg/kg/day in a single daily dose for 5 days; group 8 (NTZ)—NTZ 150 mg/kg/day for 7 days (300 mg/kg on day 1, then 150 mg/kg on days 2–7), and group 9 (control group)—pigs in the control group did not receive treatment.

An extended explanation about the basis of these treatment schemes has been presented in a previous publication.<sup>28</sup> In brief, the first three schemes were intended to evaluate the cysticidal effects of the ABZ plus PZQ as a new approach. Groups 4 ABZ and 6 PZQ simulated antiparasitic effects given in humans. The fifth group served as a positive treatment control group and received OFZ, an effective antiparasitic drug, whereas group 8 was intended to test the efficacy of NTZ. Groups 7 and 9 were non-antiparasitic treatment control groups: group 7 received PDN to rule out possible effects of steroids and group 9 did not receive any drug.

**Blood sampling.** Ten milliliters of blood were obtained from each pig by venipuncture before any treatment was given. After the onset of treatment, 5 mL blood samples were collected twice a week during the initial month of follow-up, then weekly until slaughtering with the exception of pigs in the prednisone and NTZ group, which were only sampled weekly in month 1 instead of twice a week. After sampling, the blood was centrifuged at 5,000 rpm for 10 minutes, serum samples were aliquoted into 1.5-mL vials and stored at  $-20^{\circ}\text{C}$  until processing.

**Antigen ELISA.** The antigen-detection ELISA was performed as described previously by Dorny and others.<sup>22,23</sup> This ELISA version is a slightly modified version from the original<sup>20,21</sup> and has shown sensitivity and specificity values of 86.7% and 96.7%, respectively.<sup>23</sup> All serum samples obtained from a pig during the follow-up were processed in a sole ELISA plate.

**Necropsy.** Ten weeks after treatment, all pigs were anesthetized using a combination of ketamine and xylazine and then euthanized by intravenous phenobarbital injection. Standard necropsy<sup>30</sup> was performed, and psoas and legs, the tongue, and the heart were dissected and all cysts were counted and classified by their evolution stage. Identified parasitic lesions were catalogued as viable, well defined, fluid-filled vesicles with clear fluid content; and nonviable, lesions with a preserved cystic structure but opaque or gelatinous contents, or compact, well-defined nodular lesions, or smaller, later scar stages. Brains were extracted and fixed in formalin and lesions were identified with the same criteria.

**Data analysis.** The main analysis used the total parasitic load in the carcass, considering the cysts in muscles as a practical proxy for the total parasitic burden and assuming muscle to compose approximately 25% of the pig's weight.<sup>31</sup> Since infected muscle samples and pigs had different weights, the total number of cysts per pig was estimated calculating the density of cysts per kilogram and then adjusted by the total muscle weight for each pig. In some treatment groups, there were live cysts in brain at necropsy when the effect of antiparasitic treatment in muscle, tongue, and heart was complete. To assess whether this could affect our conclusions, the analyses were repeated including brain cysts.<sup>28</sup>

The correlation between parasite burden in muscle (number of viable cysts in muscle) and circulating antigen titers at necropsy was assessed using the Pearson's test. Changes in serum antigen levels were assessed after standardization to the baseline values from each pig (ratio of the optic density [OD] value at each sampling point divided by the pretreatment OD). We modeled these relative ODs using the generalized estimating equations (GEEs) approach for panel data to account for the correlation structure of data from each pig. The relative OD was modeled in a GEE multiple regression for a Gaussian distribution, an identity link, and an exchangeable correlation structure for each animal. The direct effect of the treatment was estimated in both the rate at which the circulating antigens change (slope) as well as in the baseline level of circulating antigens (intercept). The model was also evaluated after adjusting by age and sex. A similar analysis modeled the percentage of positivity (PP), which is calculated using the OD of a known positive sample as the reference value and thus comparable across different ELISA plates. In contrast to the relative OD, the PP is not normalized.

To determine the relation between parasitic burden at necropsy and the rate of posttreatment decrease in circulating antigens, we arbitrarily classified the animals in those with no viable cysts in the carcass at necropsy ( $N = 26$ ), less than 1,000 viable cysts ( $N = 3$ ), with 1,000–10,000 viable cysts ( $N = 14$ ), and with  $> 10,000$  viable cysts ( $N = 7$ ). The effect of the parasitic burden at necropsy and its interaction with the time since the start of treatment (in days) on the relative OD of the sample (effect of the parasitic burden at necropsy in the slope and in the intercept of the kinetic curves) were estimated in a multiple GEE regression. In the subset of pigs with no viable cysts at necropsy, we also evaluated the effect of baseline parasitic burden (grossly estimated from non-viable cysts) in the rate of antigen decrease. This multivariate model considered age, sex, and the type of treatment received as covariates.

## RESULTS

**Adverse events.** From the initial 54 pigs, three pigs died during antiparasitic therapy and were excluded from the analysis. Necropsies were performed on these three pigs. One pig died at day 7 (ABZ + PZQ + PDN group) due to encephalitis caused by massive cyst destructions because this pig had approximately 140 brain cysts. Another two pigs died at day 4: one in ABZ + PDN group, from acute enteritis, and one receiving PDN alone, from pneumonia. Also the complete set of antigen results of a pig from the PZQ group was not considered because of erratic results. Finally,

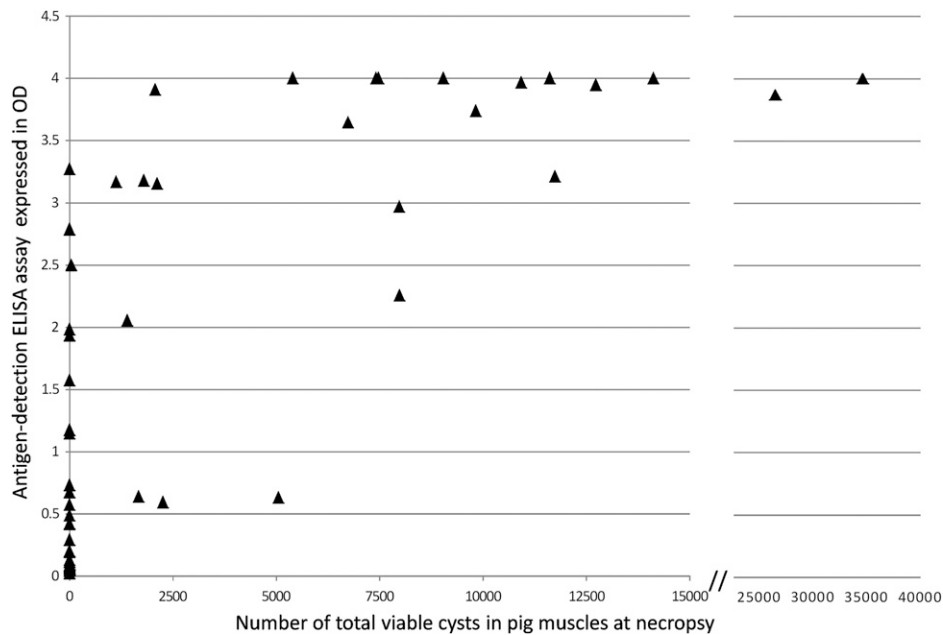


FIGURE 1. Correlation between parasitic burden and values of circulating antigen levels in naturally infected pigs.

we had a total of 50 pigs, 26 with no viable cysts at necropsy, 3 with less than 1,000 cysts, 14 pigs with 1,000–10,000 cysts, and 7 pigs with more than 10,000 cysts. There was no evidence of *Taenia hydatigena* at necropsy. A total of 693 serum samples corresponding to those 50 pigs were included in this study.<sup>28</sup>

**Parasitocidal efficacy in muscle cysts.** As previously published, the evaluation of the numbers of viable cysts in muscle, showed that the three combined schemes of ABZ plus PZQ, ABZ alone, and OFZ alone showed almost complete efficacy to kill cysts in pig muscles compared with control pigs. PZQ and prednisone had minor effects, 23.2% and 49.5% less cysts than did the control pigs, respectively, but without statistical significance, and NTZ did not show cysticidal effect. Nine pigs with no live muscle cysts at necropsy still had remaining live cysts in the brain.<sup>28</sup>

**Correlation between parasitic burden at necropsy and values of circulating antigen levels.** At necropsy, the burden of viable parasitic cysts in pig muscles was strongly correlated with its serum antigen levels (Figure 01, Pearson's  $p = 0.67$ ,  $P = 0.000$ ). Correlation increased (Pearson's  $p = 0.78$ ,  $P = 0.000$ ) after exclusion of two outlier pigs with extremely high parasitic burdens (26,510 and 36,670 cysts). Addition of number of brain cysts did not affect the association.

**Kinetics of serum antigen levels.** By the end of follow-up, serum antigen levels had decreased by five times compared with the initial values in 21 out of the 50 pigs. These were 20 of 26 pigs that were free of cysts at necropsy and 1 of 24 pigs that had persisting viable cysts (odds ratio [OR] = 76.7, 95% confidence interval [CI] = 8.1–3308.6,  $P < 0.001$ ).

Figure 2 shows the evolution of antigen levels of each pig per treatment group. GEE regression analyses after modeling both the relative OD and the PP confirmed that the rates at which the circulating antigens decrease after treatment were significantly different (negative slope) from the control, in all treatment groups ( $P < 0.01$ ) with the exception of the NTZ ( $P = 0.368$ ) and prednisone ( $P = 0.114$ ) groups.

Antigen dynamics were also examined in relation with the burden of cysts found at necropsy. Pigs were classified in four groups, without cysts, with 1–1,000 cysts, with 1,001–10,000 cysts, and with more than 10,000 cysts at necropsy. Unadjusted analysis showed that animals with less than 1,000 cysts (three animals with 8, 9, and 41 cysts) had no differences in the rate at which circulating antigens decreased ( $P = 0.17$ ) when compared with animals with no surviving cysts. However, animals with more than 1,000 cysts showed a significantly different (larger) slope ( $P < 0.001$ ), with a lower decrease rate of circulating antigens. Animals in which more than 10,000 cysts remained at necropsy did not show a significant reduction of circulating antigens during the follow-up period ( $P = 0.45$ ) (Figure 3). Multivariate adjustment by pig's age, sex, and treatment group did not affect the kinetics of antigens among groups.

Nine out of 26 pigs with no viable cysts in the muscles still had remaining live brain cysts. GEE regression analysis including those cerebral cysts in the parasitic burden at necropsy showed that the decline rate of circulating antigens in the group with 1–1,000 live cysts (now  $N = 12$ ) was significantly slower ( $P = 0.02$ ) than the antigen decline in pigs free of cysts at necropsy (now  $N = 16$ , one animal was excluded from the analysis because the brain was not examined at necropsy). Antigen decay in pigs with more than 1,000 live cysts and more than 10,000 cysts at necropsy remained significantly slower ( $P < 0.001$ ) than the rate of antigen decrease in pigs free of live cysts at necropsy. Age, sex, and treatment group were not significant and did not affect the results of kinetics of antigens among groups in the adjusted analysis.

An estimation of the baseline cyst burden in the subgroup of pigs free of viable cysts at necropsy was made, based on the numbers of degenerated or scarred lesions in the carcass. Inside this group, circulating antigens decreased faster in pigs with less than 1,000 cysts at baseline compared with animals with 1,000–10,000 cysts and animals with more than 10,000 cysts at baseline (Figure 3B). All pigs with less than 1,000 cysts at baseline had

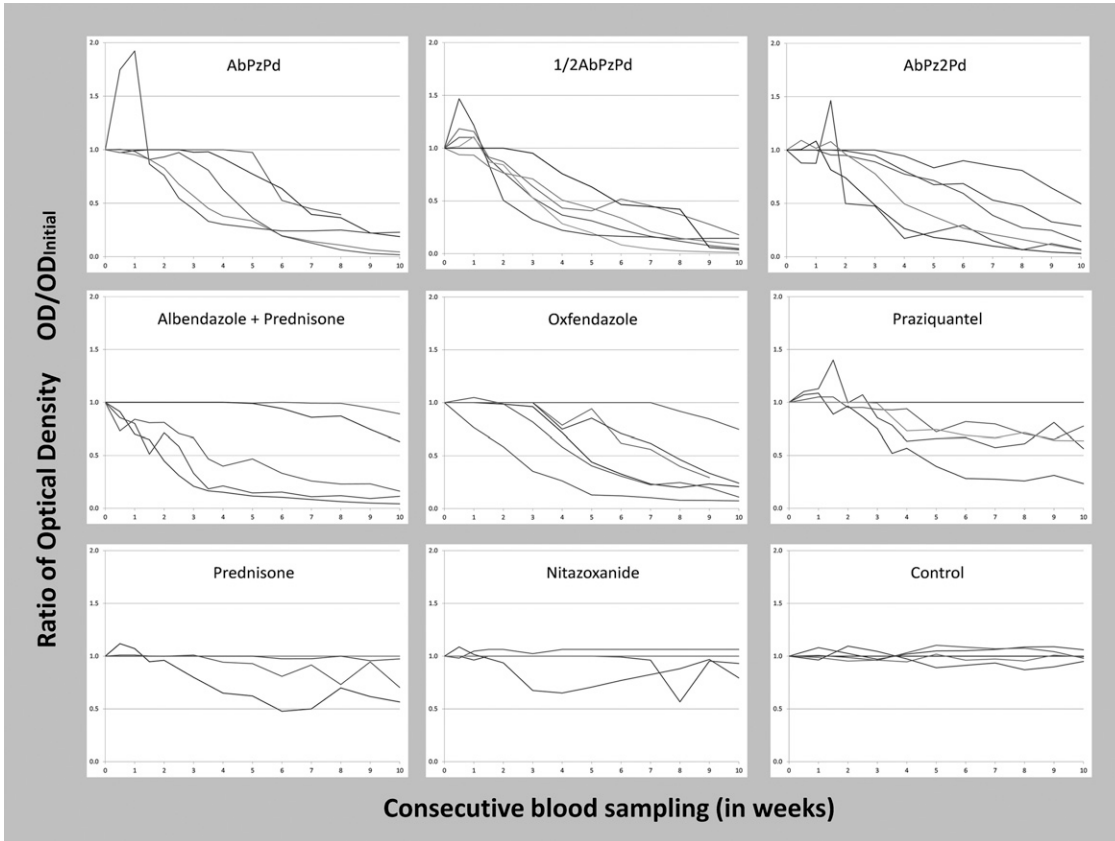


FIGURE 2. Evolution of antigen levels by treatment group in naturally infected pigs.<sup>28</sup> Albendazole (Ab), praziquantel (Pz), prednisone (Pd).

decreased their OD values by half by week 4 (6/6), compared with only 6/13 of those with 1,000–10,000 cysts ( $P = 0.45$ ), although the mean slope was not significantly different ( $P = 1.08$ ). In the group of pigs with more than 10,000 cysts at

baseline, none of them reduced 50% of their OD levels by week 4 (0/7), and the slope of decrease was significantly larger (slower antigen decrease) than in pigs with less than 1,000 cysts ( $P = 0.021$ ).

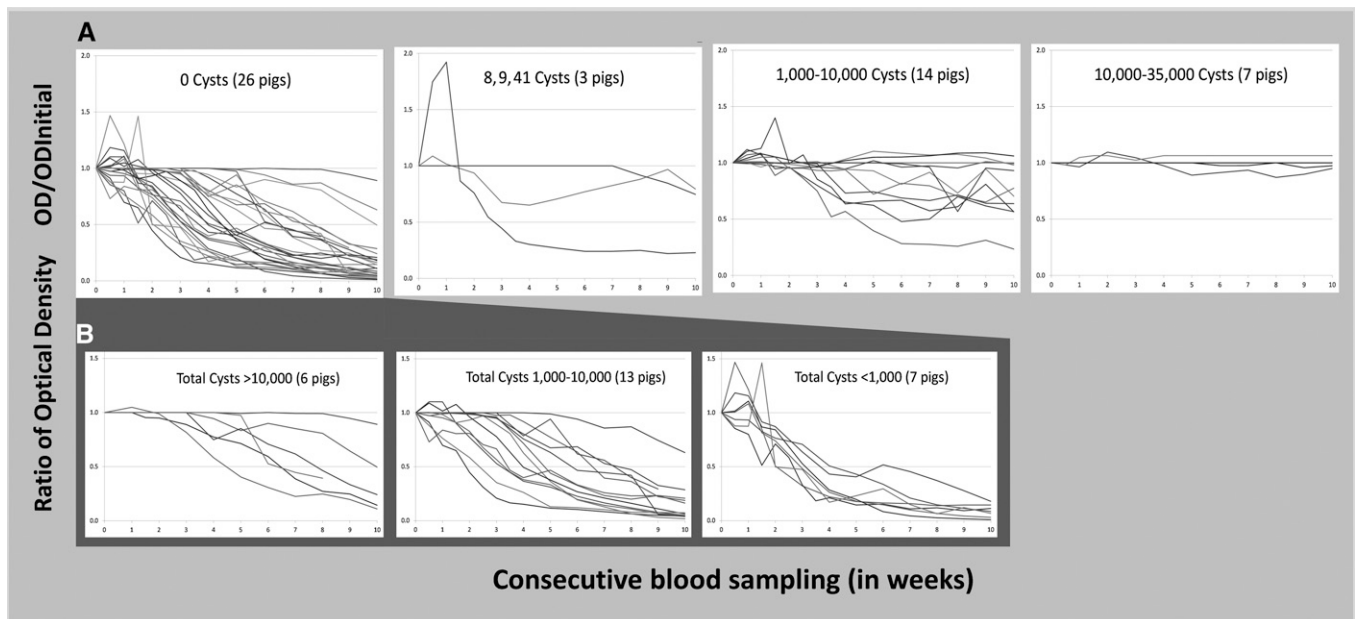


FIGURE 3. (A) Evolution of antigen levels stratified by parasitic burden at necropsy. (B) Evolution of antigen level of pigs with no viable cysts in the carcass at necropsy stratified by estimated parasitic burden before treatment.

## DISCUSSION

Serial posttreatment follow-up of serum antigen levels in a *T. solium*-infected pig population demonstrated associations between antigen levels, parasite burden, and efficacy of antiparasitic treatment. As expected, there was a correlation between antigen levels and cyst burden, as previously reported in human and porcine NCC.<sup>20,25,32</sup> Antigen levels dropped in pigs that had fewer cysts at necropsy and the decrease in antigen levels was consistent with treatment efficacy.

Antigen levels decreased rapidly in response to successful antiparasitic treatment. This finding may have implications in the management of human NCC. If serum antigen kinetic is similar in humans, more effective antiparasitic treatment should result in faster or more marked decreases in circulating antigen levels, likely measurable few weeks after treatment onset. The levels of circulating antigen and their rate of decay were also affected by the numbers of degenerated cysts or scars at necropsy, likely as a proxy for the initial cyst burden. A higher initial parasitic burden was associated with slower clearance of circulating antigens.

The antiparasitic effect was stronger in muscles than brain. Surviving brain cysts were found in nine of 26 pigs where the cysticidal effect in muscle was complete. Similar findings have been reported previously.<sup>33–35</sup> In this cohort of heavily infected pigs, the dynamics of antigens were mostly driven by muscle cysts. The trend of decline in circulating antigen by groups, both according to estimated burden before treatment and number of viable cysts at necropsy, did not change when cerebral brain cysts were included in the analysis. The circulating antigen levels at necropsy in the nine pigs with only remaining viable cyst in the brain were not different from those of the 16 pigs in which the antiparasitic treatment cleared both muscle and brain cysts.

Interestingly, most of the pigs allocated the treatment groups receiving PZQ had an increment in antigen OD levels shortly after treatment as shown in Figure 2. This was not the case in pigs treated with benzimidazoles. This increase may result from the mode of action of PZQ that causes tegumentary damage among other effects, perhaps resulting in greater antigen exposition than that caused by benzimidazoles, which have a less aggressive mode of action (selective degeneration of parasite cytoplasmic microtubules, leading to decreased adenosine triphosphate formation and energy depletion).<sup>36,37</sup> However, this early increment in antigen OD levels was not predictive of antiparasitic efficacy.

Naturally infected pigs differ from humans with NCC in a series of characteristics and thus its use as a model should be interpreted in this context. Given the short lifespan of pigs (usually less than a year), porcine infections are likely much more recent than brain cysts in humans. The predominance of viable muscle cysts also argues for a more recent infection. Since the large case series of NCC in British soldiers returning from India, it is well known that brain cysts may survive for several years after muscle cysts have resolved.<sup>38</sup> Tongue-positive pigs more likely represent a heavily infected subgroup since in field settings, most infected pigs have less than 10 cysts in the entire carcass.<sup>39</sup> As much as it is known, most humans with NCC have no evidence of viable muscle cysts by the time of diagnosis (although a significant proportion will have muscle calcifications).<sup>40</sup> From the above reasons, it follows that antigen levels in tongue positive pigs are

likely higher than those in humans with parenchymal NCC and consequently changes detected in this model may not be so easy to determine in clinical cases of parenchymal NCC. Extraparenchymal NCC, on the other hand, presents very high antigen levels, which have already been shown to reflect the evolution of the disease.<sup>14,15,27,41</sup>

Antigen detection by a monoclonal antibody-based ELISA is a reliable tool to evaluate the response to antiparasitic treatment in the porcine model and may prove useful to monitor the efficacy of antiparasitic treatment in human NCC, guiding medical decisions along patient follow-up.

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